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Long Term Stability of Volatile Nitrosamines in Human Urine

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Abstract

Volatile nitrosamines (VNAs) are established teratogens and carcinogens in animals and classified as probable (group 2A) and possible (group 2B) carcinogens in humans by the IARC. High levels of VNAs have been detected in tobacco products and in both mainstream and sidestream smoke. VNA exposure may lead to lipid peroxidation and oxidative stress (e.g. inflammation), chronic diseases (e.g. diabetes), and neurodegenerative diseases (e.g. Alzheimer's disease). To conduct epidemiological studies on the effects of volatile nitrosamine exposure, short-term and long-term stabilities of VNAs in urine matrix are needed. In this report, the stability of six VNAs (N-nitrosodimethylamine, N-nitrosomethylethylamine, N-nitrosodiethylamine, N-nitrosopiperidine, N-nitrosopyrrolidine, and N-nitrosomorpholine) in human urine is analyzed for the first time using *in vitro* blank urine pools spiked with a standard mixture of all six VNAs. Over a 24 day period, analytes were monitored in samples stored at approximately 20°C (collection temperature), 4–10°C (transit temperature), and –20°C and –70°C (long-term storage temperatures). All six analytes were stable for 24 days at all temperatures (n = 15). The analytes were then analyzed over a longer time period at –70°C; all analytes were stable for up to one year (n = 62). A subset of 44 samples was prepared as a single batch and stored at –20°C, the temperature at which prepared samples are stored. These prepared samples were run in duplicate weekly over 10 weeks, and all six analytes were stable over the entire period (n = 22).

Keywords

volatile nitrosamines; short-term and long-term stability; human urine

Introduction

The potential for volatile nitrosamine exposure in the general population is a growing concern. Volatile nitrosamine (VNA) formation can occur from a wide variety of everyday sources, including cured meats, cosmetics, contaminated drinking water, and tobacco products, both in the products themselves as well as in mainstream and sidestream smoke

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*The findings and conclusions in this study are those of the authors and do not necessarily represent the views of the U.S. Department of Health and Human Services, or the U.S. Centers for Disease Control and Prevention. Use of trade names and commercial sources is for identification only and does not constitute endorsement by the U.S. Department of Health and Human Services, or the U.S. Centers for Disease Control and Prevention.

[1–12]. VNAs are also formed endogenously in the acidic conditions of the stomach through the nitrosation of secondary and tertiary amines via nitrite interaction [2, 3, 13–18]. VNAs are classified as group 2A and 2B carcinogens by the International Agency for Research on Cancer (IARC) and have been linked to tumorigenesis in the liver, lungs, kidney, bladder, pancreas, and esophagus [1–3, 13–15, 18–20]. VNAs have also been associated with lipid peroxidation, oxidative stress, chronic diseases such as diabetes, and neurodegenerative diseases like Alzheimer's disease [21–28]. Exposure to VNAs can be assessed by measuring VNAs in urine. The six VNAs monitored in this study are N-nitrosodimethylamine (NDMA), N-nitrosomethylethylamine (NMEA), N-nitrosodiethylamine (NDEA), N-nitrosopiperidine (NPIP), N-nitrosopyrrolidine (NPYR), and N-nitrosomorpholine (NMOR) (Figure 1).

In order to ensure the validity of analytical results in large studies such as the National Health and Nutrition Examination Survey (NHANES), the stability of the analytes being monitored must be documented. Here we monitored both the short-term (24 days) and long-term (one year) stability of all six VNAs in spiked human urine. The short-term stability study covered a range of temperatures at which the samples are stored during collection and transport: samples are typically collected at room temperature (approximately 20°C), transported in chilled shippers which can range from 4°C to 10°C, and, once delivered to the destination laboratory, placed in short-term (–20°C) or long-term (–70°C) storage. This whole process can take anywhere from one to three weeks, depending on the originating location of the sample. Long-term stability samples were stored only at –70°C. The stability of prepared samples was also monitored in this study; this is imperative to ensure the validity of samples yet to be analyzed due to instrument downtime or re-injected samples in case of instrument failure or interruption. These prepared samples were stored at –20°C, the temperature at which samples yet to be analyzed or re-injected are stored. The resulting data will be used to design and validate procedures for collecting, transporting, and storing urine specimens for subsequent VNA analysis as part of large population studies.

Methods and Materials

Materials

Native standard stock purchased from Supelco (Sigma-Aldrich, St. Louis, MO) was a 2 mg/mL mixture of all 6 native analytes in dichloromethane (DCM). Deuterium-labeled internal standards NDMA-d₆, NDEA-d₁₀, NPYR-d₈, and NMOR-d₈ were purchased individually in DCM from Cambridge Isotope Laboratories (Tewksbury, MA); NMEA-d₃ and NPIP-d₁₀ were purchased individually as oils from Toronto Research Chemicals (Toronto, Canada). Methanol (MeOH), dichloromethane (DCM), and acetonitrile (ACN) were HPLC grade, purchased from Honeywell Burdick & Jackson (Muskegon, MI). Sample plates were Axygen 48-well plates with a 5 mL well capacity. GC vials were Wheaton 11 mm amber crimp vials with a 300 µL insert; crimp caps were SUN-SRi 11 mm aluminum crimp caps with rubber septum. All GC-QQQ parts were purchased from Agilent Technologies (Santa Clara, CA).

QC Pools

Two fresh urine QC pools were created from anonymously collected urines by spiking with the six VNAs: QC low at 50 pg/mL (500 µL of a 100 pg/µL methanol stock in 1000 mL urine) and QC high at 200 pg/mL (2000 µL of a 100 pg/µL methanol stock in 1000 mL urine). The pools were aliquoted in 2.2 mL aliquots into 2.5 mL Nalgene cryovials and stored at the following temperatures: 20 samples each at −20°C, 4°C, 10°C, and room temperature (approximately 20°C), and the remaining samples (approximately 370) at −70°C. Blank urine pools were tested prior to spiking to ensure no contributing interferences were detected during GC-QQQ analysis.

Automated Sample Preparation

The automated sample preparation method used for this study was developed and validated by this group [29]. Urine samples were transferred from cryovials in 2 mL aliquots into a 48-well plate and spiked with 500 pg of internal standard using a Hamilton Star liquid handling system. A liquid-liquid extraction was performed using the Caliper Staccato workstation, after which samples were concentrated via solvent evaporation, manually transferred to GC vials, and further concentrated using acetonitrile as a keeper solvent. Sample vials were sealed and transferred to the GC-QQQ for analysis.

GC/MS-MS (QQQ) Analysis

All analyses were performed on an Agilent 7890-7000C GC-MS/MS (QQQ). The 7890 GC was equipped with a multimode inlet (MMI) and a single taper helix liner. The injection volume was 5 µL. The initial inlet temperature was 5°C, which was held for 0.85 minutes after injection and then heated at 600°C/min to 300°C. A programmed temperature vaporization (PTV) solvent vent mode was used, venting at 200 mL/min flow rate and 5 psi pressure for 0.7 minutes after injection. A two-column setup connected by a backflush union was used for the GC, with helium as the carrier gas. The first column was an Agilent DB-WAXetr (30 m × 0.25 mm × 0.5 µm) with a constant flow rate of 3 mL/min for the first 1.3 minutes, followed by a 1.2 mL/min flow rate for the remainder of the run. The second column was deactivated fused silica (1 m × 0.15 µm) with a constant pressure of 1 psi. The GC oven was initially set to 35°C for 1 minute after injection then heated at 20°C/min to 245°C. A backflush was performed for 5 minutes post-run, with a −1.9795 mL/min constant flow for the first column, a 25 psi constant pressure for the second column, and an oven temperature of 250°C. The transfer line temperature and MS source were both set to 250°C. The 7000C MS source mode was positive chemical ionization (CI) with ammonia (blue grade) as the CI gas; ultra-high purity nitrogen was used as the collision gas.

Results

Short Term Stability of Analytes in Human Urine at Various Temperatures

Samples stored at all 5 temperatures were prepared and run in 15 batches over a 24-day period. During this time, no statistically significant decrease in concentration was observed for any of the analytes in either QC pool at all storage temperatures (n = 15) (Figures 2a–c, Supplemental Figures 1a–c). The coefficients of variation (CVs) for all analytes in both

pools are below 10%, with CVs for all analytes in the 200 pg/mL QC pool below 5% (Supplemental Table 1).

Long Term Stability of Analytes in Sample at -70°C

Samples stored at -70°C were run in duplicate past the duration of the short term stability experiment up to 358 days, with 31 averaged data points total over this period. No statistically significant decrease in concentration was observed for any analyte ($n = 62$) (Figures 3a–c, Supplemental Figures 2a, 2c). The CVs for all analytes in both pools are below 20%, with CVs for all analytes in the 200 pg/mL QC pool below 12% (Supplemental Table 2). The increase in CV between the long term and short term stability samples can be attributed to variances in the composition of each analytical column used over the duration of the study. Particularly with NPIP and NPYR, variable levels of interference were observed in the respective chromatogram windows depending on the individual column, leading to fluctuations in the final calculated concentrations.

Stability of Analytes in Prepared Samples at -20°C

To monitor the stability of prepared samples, 22 QC samples at each concentration were prepared on day 1 and run in duplicate weekly for 10 weeks, yielding 11 averaged data points for each analyte per QC pool. No statistically significant decrease was observed in any analyte in either pool ($n = 22$) (Figures 4a–c, Supplemental Figures 3a–c). The CVs for all analytes in both pools are below 10%, with CVs for all analytes in the 200 pg/mL QC pool below 6% (Supplemental Table 3).

Discussion

Samples in large studies such as the National Health and Nutrition Examination Survey (NHANES) are collected over several months, shipped at varying temperatures, and stored for years before and after analysis. Therefore, the stability of the analytes being monitored must be maintained in order to validate any data generated. The short-term stability results show that measurement of VNAs (N-nitrosodimethylamine, N-nitrosomethylethylamine, N-nitrosodiethylamine, N-nitrosopiperidine, N-nitrosopyrrolidine, and N-nitrosomorpholine) remain stable under various storage conditions, from sample collection in the field (20°C) to typical transit time and conditions ($4\text{--}10^{\circ}\text{C}$) for up to 24 days. This time period allows for sample collection in remote locations to be shipped back to final laboratories for analyses. In addition, measurement of these six VNAs in human urine are valid at least up to 1 year if samples are frozen at -70°C or lower. In prepared urine samples, these six VNAs are stable for up to 10 weeks at -20°C . This relatively long storage time of prepared samples is useful in case of instrument downtime.

This study only concerned itself with the thermal stability of volatile nitrosamines. There are of course other factors influencing the overall stability of the analytes. However, volatile nitrosamines are rather stable compounds and difficult to degrade once formed. Denitrosation only occurs in $>1\text{M}$ strong acid solutions, and nitrosamines are otherwise stable at neutral and basic pH [12]. The average pH of human urine is approximately 6.2, with a range of 4.5–8.0 depending on factors such as time of day collected (night vs day

urine), dietary factors, and bacterial presence [30–32]. Only the most extreme samples might reach conditions suitable for VNA denitrosation, and these samples would be rejected from the study on those grounds. Nitrosamines will degrade with prolonged exposure to ultraviolet light, but this factor should be limited or nonexistent during collection and transport [12].

The long-term stability of these six VNAs will be continued in our laboratory for several years to determine if measurements of VNAs in old residual samples will be valid.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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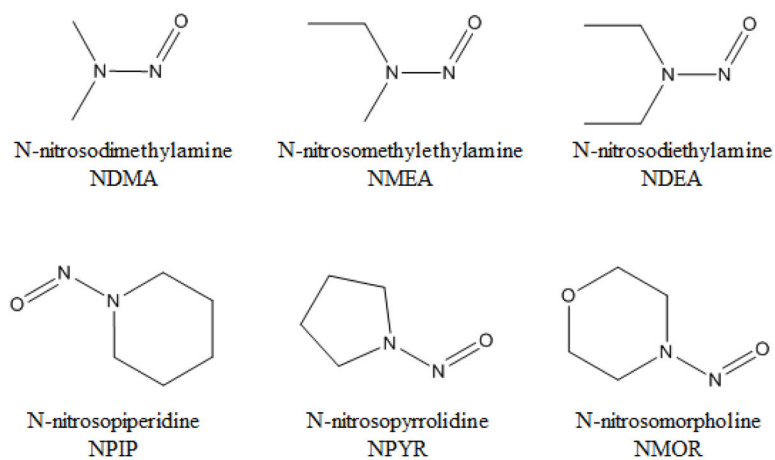


Figure 1.
Structures of Volatile Nitrosamines

Figure 2a

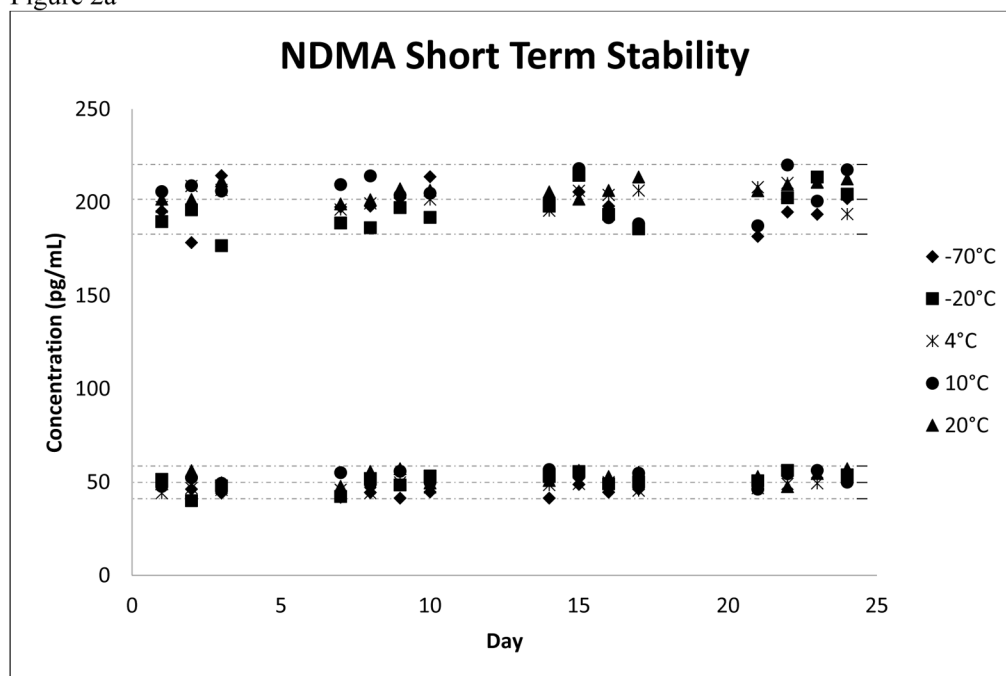


Figure 2b

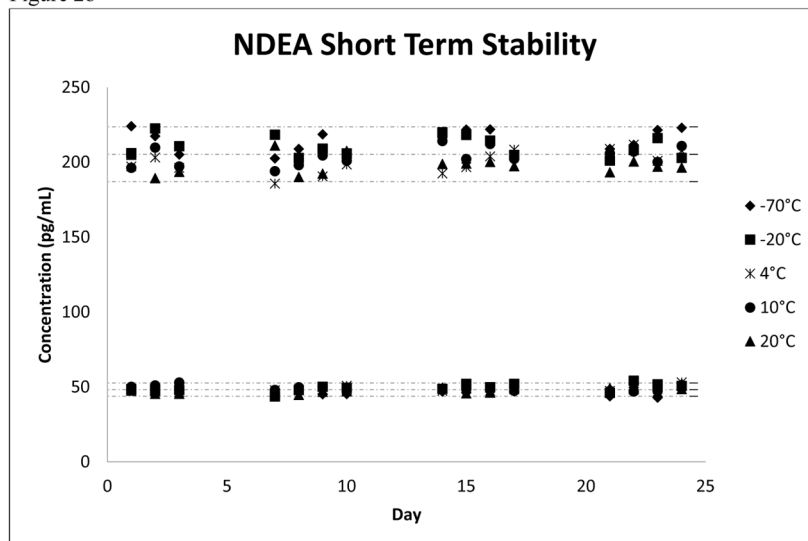


Figure 2c

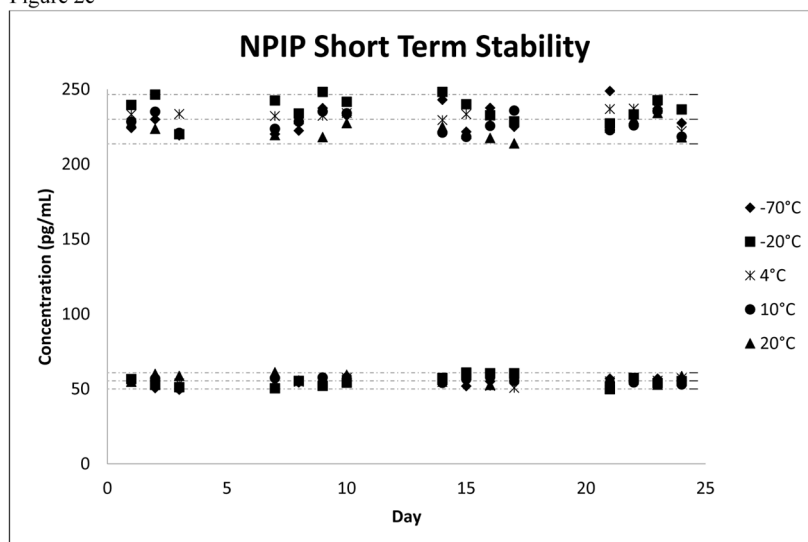
**Figure 2.**

Figure 2a – Short Term Stability of NDMA in Human Urine at Various Temperatures

Figure 2b – Short Term Stability of NDEA in Human Urine at Various Temperatures

Figure 2c – Short Term Stability of NPIP in Human Urine at Various Temperatures

*Dashed lines denote mean \pm 2 standard deviations; QC low = 50 pg/mL, QC high = 200 pg/mL

Figure 3a

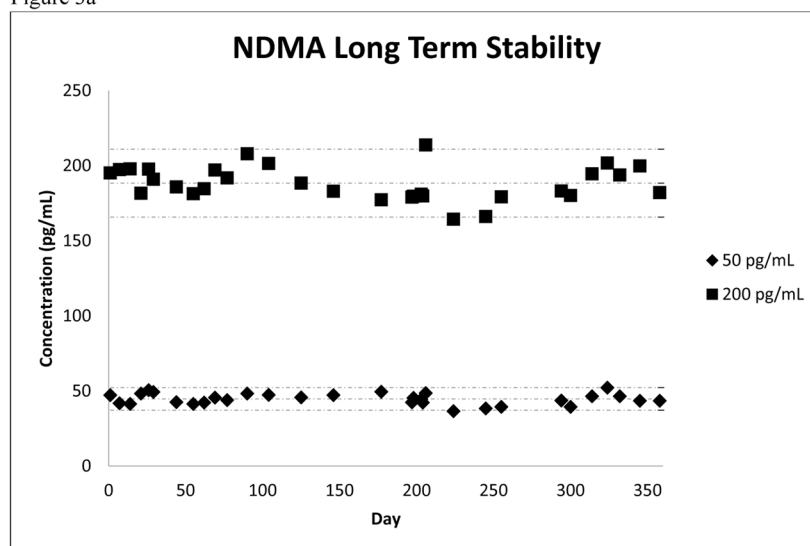


Figure 3b

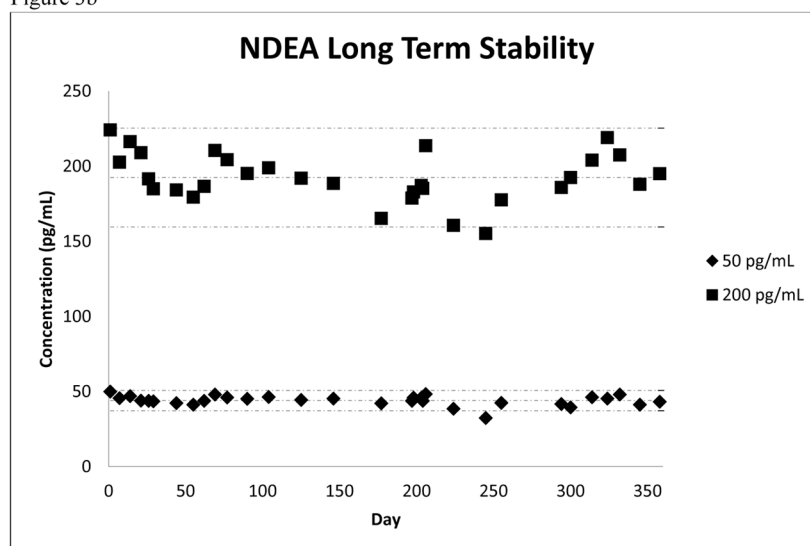


Figure 3c

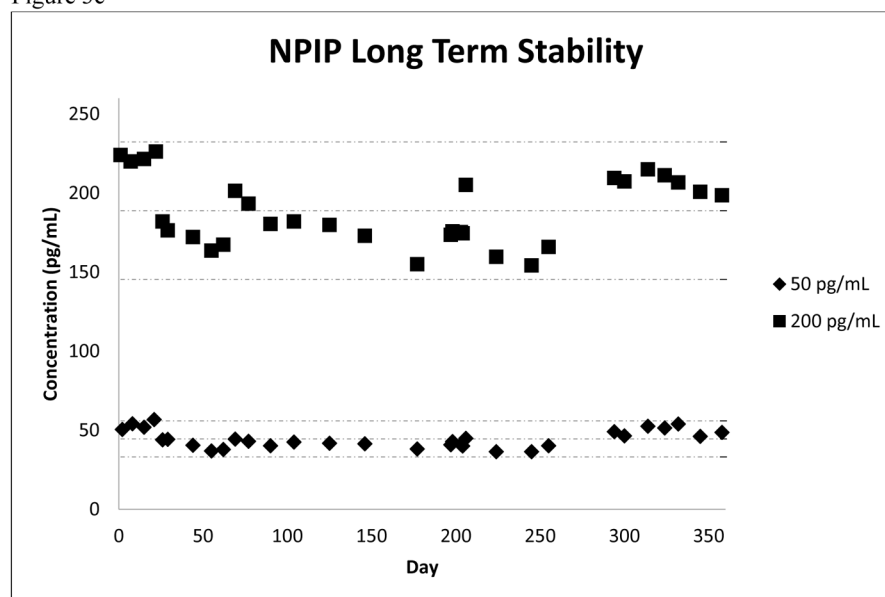
**Figure 3.**Figure 3a – Long Term Stability of NDMA in Human Urine at -70°C Figure 3c Long Term Stability of NDEA in Human Urine at -70°C Figure 3d – Long Term Stability of NPIP in Human Urine at -70°C *Dashed lines denote mean \pm 2 standard deviations

Figure 4a

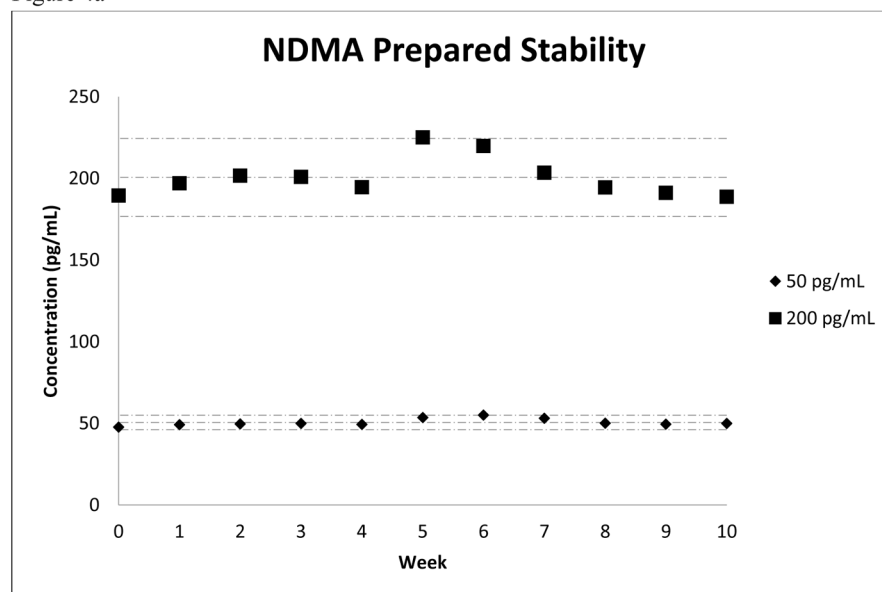


Figure 4b

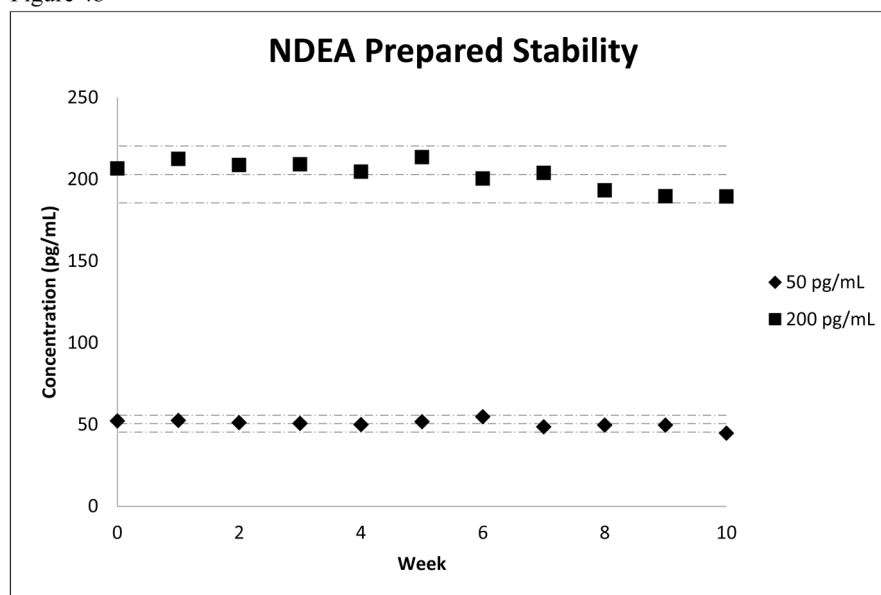
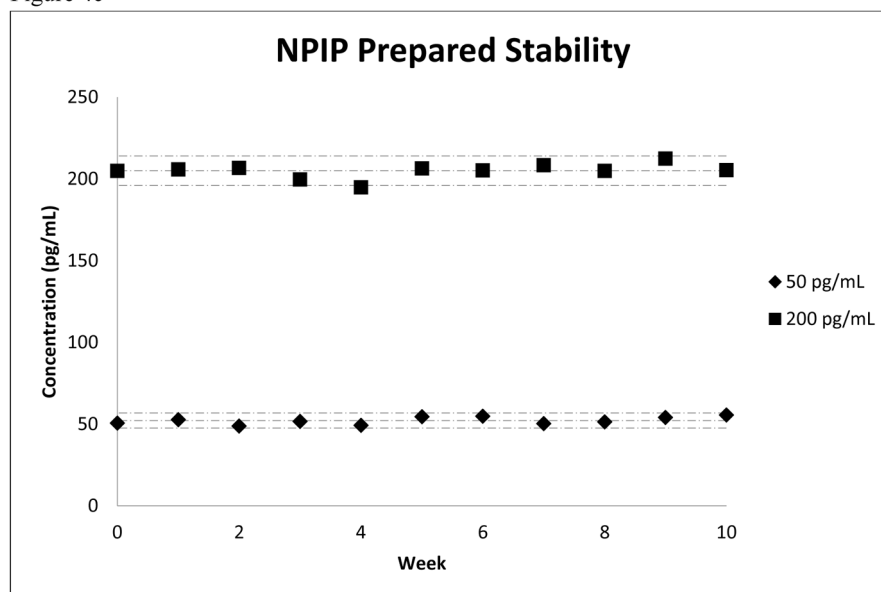


Figure 4c

**Figure 4.**Figure 4a – Stability of NDMA in Prepared Samples at -20°C Figure 4c – Stability of NDEA in Prepared Samples at -20°C Figure 4d – Stability of NPIP in Prepared Samples at -20°C *Dashed lines denote mean \pm 2 standard deviations